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Liposome-mediated delivery of DNA to carrot protoplasts

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Abstract. The encapsulation of DNA within liposomes and subsequent fusion of the liposomes with carrot (*Daucus carota* L.) protoplasts were examined to determine optimum conditions for effective liposome-mediated delivery of DNA to protoplasts. *Escherichia coli* [^{3}H]DNA could be encapsulated with 50% efficiency using encapsulation volumes as low as 0.5 ml. Incorporation of liposome-encapsulated [^{3}H]DNA by carrot protoplasts increased linearly for 2.5 h, and increasing the ratio of protoplasts to liposomes increased the total amount of radioactive label incorporated within the protoplasts. Liposome-mediated incorporation of [^{3}H]DNA by protoplasts increased over a range of polyethylene glycol concentrations up to 20%, but Ca^{2+} did not increase liposome-mediated incorporation when present in the liposome-protoplast incubation mixture. Optimum incorporation was observed when the pH of the liposome-protoplast incubation medium was decreased to 4.8. Encapsulation experiments using DNA of the plasmid pBR322 indicated that an average of 200–1,000 intact copies of pBR322 were sequestered within each nucleus after liposome delivery.

Key words: *Daucus* – DNA transfer – Lipid vesicle transfer – Liposome transfer – Protoplasts.

Introduction

In the last several years liposomes have been used to insert a wide variety of molecules into mammalian cells (Weissmann et al. 1975; Papahadjopoulos et al. 1974; Cohen et al. 1976). Several laboratories have demonstrated the liposome-mediated insertion of mRNA into cells and subsequent translation of the mRNA by those cells. Ostro et al. (1978) and Dimitriadis (1978) encapsulated globin mRNA within liposomes and inserted it into non-globin-producing cell lines. The delivered mRNA was translated by the cells and the protein product was isolated. Furthermore, transfer of mammalian genes from one cell type to another using lipid-coated chromosomes has been demonstrated by Mukerjee et al. (1978). Metaphase chromosomes from hypoxanthine-guanine-phosphoribosyltransferase(HGPRT)-positive cells were encapsulated within liposomes and fused with HGPRT-negative cells. The gene coding for HGPRT and two other linked genes were transferred to the HGPRT-negative cells.

Little work has been done using plant protoplasts as the recipient to study liposome-mediated delivery of macromolecules. Matthews et al. (1979) have demonstrated the ability of liposomes to deliver high-molecular-weight RNA to carrot protoplasts. *E. coli* [^{3}H]RNA was encapsulated within liposomes which were then fused with carrot protoplasts. Liposomes protected the internally sequestered RNA from nuclease degradation. [^{3}H]RNA recovered from washed protoplasts indicated that the 23S RNA fraction was degraded while portions of the 16S and 4S RNA fractions were recovered intact from within the protoplasts. Several investigators have demonstrated that liposome encapsulation of DNA protects the DNA from external DNase degradation (Hoffman et al. 1978; Mannino et al. 1979; Dimitriadis 1979). Lurquin (1979, 1981) has encapsulated [^{3}H]pBR322 plasmid within liposomes and fused them with protoplasts. Nuclei harvested from the protoplasts in the presence of a large excess of unlabelled DNA contained pBR322 DNA, some of which was in a relatively undegraded form. Using autoradiographic techniques, Rollo et al. (1981) also demonstrated liposome-mediated transfer of DNA to plant protoplasts.

Several parameters have been reported to influence the insertion of RNA into mammalian cells (Ostro et al. 1980). Uchimiya (1981) has examined the effects of the ratio of liposomes to protoplasts on

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liposome delivery of fluorescein diacetate to plant protoplasts. In this paper we examine a variety of parameters affecting liposome-protoplast interaction, to optimize these parameters for maximum insertion of DNA into plant protoplasts. These parameters include temperature, pH and use of polyethylene glycol (PEG).

Material and methods

Preparation and maintenance of protoplasts. Garden carrot (*Daucus carota* L. cv. Danvers: Northrup King Seeds, Minneapolis, Minn., USA[1]) root cells were grown in 50 ml of defined medium (MS) containing 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), as described in Matthews and Widholm (1978). Cultures were inoculated with 0.5 g fresh weight of cells and were used in experiments on the fifth day of culture. Protoplasts were prepared from the carrot cell-suspension cultures by incubating the cells for 4 h in 4% Cellulysin (Calbiochem, San Diego, Cal., USA), 2% Macerase (Calbiochem), 0.4 M sorbitol, pH 6.0. The protoplasts were washed two times with the same medium lacking enzymes. Cell-wall formation and cell division was examined by plating cells in the liquid medium described by Dudits et al. (1977) for carrots. In some experiments the medium of Slavik and Widholm (1978) was used. It contains the same concentration of salts as the MS medium with the following additions (mg/l): 150 xylose, 150 arabinose, 100 *myo*-inositol, 100 glucose, 34,000 sucrose, 45,000 sorbitol, 0.01 dimethylallyladenine (Sigma Chemical Co., St. Louis, Mo., USA), and 0.1 2,4-dichlorophenoxyacetic acid (2,4-D) with the pH adjusted to 6.0.

Preparation of liposomes containing E. coli [^{3}H] DNA. Large, unilamellar and oligolamellar liposomes were prepared using the reverse-phase evaporation technique described by Szoka and Papahadjopoulos (1978) with slight modifications, Lecithin (L) (soybean Type III-S; Sigma), dicetyl phosphate (D) (Sigma) and lysolecithin (Ly) (Sigma) were dissolved in chloroform in an 8L:2D:0.4Ly ratio and added to a 50-ml round-bottom flask. The solvent was rotary-evaporated under reduced pressure, leaving a lipid coating on the bottom of the flask. The lipid was redissolved in 5 ml diethyl ether. The aqueous phase (1.5 ml unless otherwise stated) containing chromosomal [^{3}H]DNA from *E. coli* (New England Nuclear, Boston, Mass., USA) or plasmid pBR322 in 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (HEPES), 0.7 M NaCl, 0.7 M KCl (pH 7.4) was added directly to the organic phase and nitrogen was added. The two-phase system was sonicated for 3 min, or in later experiments, vigorously handshaken for 10 min. The mixture was placed on the rotary evaporator and the organic solvent was removed under reduced pressure (200 rpm for 20 min). Another 1 ml of the aqueous phase was added and rotary evaporation was continued for another 10–15 min. The liposomes were removed from the flask, incubated for 45 min at room temperature with 20 μg/ml DNase (Sigma) and then incubated for 1 h with predigested Pronase as described previously (Matthews et al. 1979). This suspension was diluted by adding 2 ml HEPES buffer and then filtered through Sepharose 4B (Pharmacia, Uppsala, Sweden) to remove unencapsulated, DNase-degraded [^{3}H]DNA from the liposome-encapsulated DNA. The chromatographed liposome preparations had an A_{650} ranging from 0.6 to 1.6 depending upon the amount of lipid used during encapsulation, and contained between 10,000 and 30,000 cpm of [^{3}H]DNA/ml. The A_{650} readings were similar among preparations of liposomes containing the same amount of lipid. The A_{650} of the liposomes upon dilution is linear through an A_{650} of 1.6.

Incubation of carrot protoplasts with liposomes. Unless stated otherwise, carrot protoplasts (1 ml, $5.5 \cdot 10^5$) were incubated at 32° C with 1 ml liposomes ($A_{650}=0.6$) and 1.5 ml of 0.8 M mannitol to maintain protoplast stability. Deviations from these conditions for individual experiments are described in the text. The liposomes and protoplasts were incubated in screwcapped test tubes, while being rotated at 0.5 rpm on a rotating apparatus for 1.5 h. After the incubation, the cells were washed four times and analyzed for radioactivity. The samples were counted in Aquasol 2 scintillation cocktail (New England Nuclear) using a liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill., USA).

In experiments using polyethylene glycol (PEG) (Sigma; MW=8,000), the liposome-protoplast mixture was incubated as described above. Polyethylene glycol was then added at the concentrations stated in the text, and the preparation incubated at room temperature for 1.5 min. Then 4 ml of 0.1 M glucose and 2 ml of 0.4 M mannitol were slowly added to the mixture. After gently inverting the tubes several times to ensure proper mixing, the protoplasts were sedimented by centrifugation at 200 *g* for 5 min. The supernatant was removed and the protoplasts were washed with mannitol as described above.

Analysis of DNA sequestered in the nuclear fraction. After optimizing several parameters for the encapsulation and delivery of DNA by liposomes, the fate of plasmid pBR322 DNA was determined after encapsulation and delivery to protoplasts. Protoplasts were prepared using solution A: 2% Cellulysin, 0.5% Macerase, 0.3 M sorbitol, 0.3 M mannitol, 3 mM 2-(N-morpholino)ethanesulfonic acid (MES), 6 mM $CaCl_2$ and 0.7 mM NaH_2PO_4 (pH 5.7). The washing solution was the same as solution A, except it lacked the enzymes. Protoplasts were added to liposomes containing 60 μg pBR322 in a fusion mixture which was the same as the washing solution but adjusted to pH 5.2. Protoplasts were incubated 2 h at 37° C while rotating at 2 rpm. Liposomes (1.0 ml, $A_{650}=0.6$) were incubated with 10^6 protoplasts in a final volume of 2.2 ml.

After incubation, the protoplasts were centrifuged and the liposomes in the supernatant were discarded. After two more washings to remove unfused liposomes, the protoplasts were suspended in TM buffer [0.12 M Tris-HCl, 0.001 M $CaCl_2$, 0.001 M $ZnCl_2$, 0.001 M $MgCl_2$ (pH 7.0) (Maio and Schildkraut 1967), with 1% Triton X-100] in the presence or absence of excess carrier DNA (200 μg). The solution was incubated on ice for 5 min, rapidly swirled for 1 min using a cyclo-mixer (Clay Adams, Parsippany, N.J., USA), then passed two times through a 22-gauge (outer diameter=0.71 mm) needle to release the nuclei. The solution was centrifuged for 2 min at 100 rpm to remove cells and debris. The supernatant, containing nuclei, was layered on 1.8 M sucrose and centrifuged at 4° C at 10,000 rpm for 10 min. The pellet contained nuclei uncontaminated by cells.

The nuclei were resuspended in 10 mM Tris-HCl (pH 7.0), 5 mM $CaCl_2$, 10 mM $MgCl_2$; treated with 5 μg/ml DNase (Worthington, DPFF, Freehold, N.J., USA) at 25° C for 15 min; washed five times with 10 mM Tris (pH 8.0), 5 mM $CaCl_2$, 5 mM mercaptoethanol, and 0.1% Triton X-100; and resuspended in 0.2 M Tris (pH 8.0), 0.1 M ethylenediamine tetraacetic acid (EDTA). They were then lysed by the addition of sodium dodecyl sulfate (SDS) to 1% (w/v) and the lysate was incubated with proteinase K (Merck, Rahway, N.J., USA) at 65° C for 20 min. Potassium acetate was added to 1.2 M and incubation continued on ice for 30 min. Following centrifugation at 12,000 *g* for 15 min the supernatant, containing DNA, was precipitated with two volumes of

[1] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable

ethanol at −20° C overnight and redissolved in 10 mM Tris (pH 8.0), 1 mM EDTA. DNA was size-fractionated by electrophoresis in Tris-acetate buffer (McDonnell et al. 1977) through a 1% agarose gel, and transferred to nitrocellulose (Southern 1975) after depurination and denaturation (Wahl et al. 1979). Hybridizations of the immobilized DNA on nitrocellulose to pBR322 probe DNA were carried out for 24–48 h in 6 times SSC (SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), five times Denhardt's reagent (Denhardt 1966) with 0.5% SDS. pBR322 probe DNA was labelled with [α-^{32}P]dCTP ($\geq$400 Ci/mmol; Amersham Inc., Arlington Heights, Ill., USA) by nick translation (Rigby et al. 1977) to a specific activity of $1 \cdot 10^8$ cpm/μg. The dried filters were autoradiographed using Kodak X-Omat R film (Eastman Kodak Co., Rochester, N.Y., USA).

Results

Optimization of liposome-protoplast fusion. The uptake of liposomes by carrot protoplasts occurred in a nearly linear fashion for the first 2 h as determined by percent incorporation of the ^{3}H-label. The rate of uptake decreased steadily thereafter and very little additional uptake occurred after 8 h. After treatment with liposomes, the carrot protoplasts were plated in agar to determine if the liposome treatment was toxic. The plating efficiencies of the control protoplasts and liposome-treated protoplasts were similar. Approximately 10–15% of the protoplasts formed a cell wall and underwent sustained cell division. Comparable plating efficiencies of carrot protoplasts have been reported by Dudits et al. (1976) using liquid culture.

Addition of polyethylene glycol (PEG) to the incubation mixture containing protoplasts and liposomes caused no increase in the amount of incorporation of liposomes during a 2-min incubation period. However, if the liposome-protoplast mixture was incubated for 1.5 h prior to PEG addition, there was a large increase in the amount of liposome incorporation (Fig. 1). Low PEG concentrations appeared to have no effect on the liposome-protoplast interaction. At high PEG concentrations (18%), some of the protoplasts were ruptured and many of the protoplasts fused together. Protoplast damage was less if a small amount of liposomes and protoplasts were used and when centrifugation to remove PEG was eliminated. However, extensive protoplast fusion still occurred. Because more delicate procedures used in protoplast-protoplast fusion studies could not provide the amount of materials necessary for a quantitative study of incorporation these procedures were not used.

Ca^{2+} has been used in liposome-liposome aggregation studies to study membrane interactions (Poste and Allison 1973; Papahadjopoulos et al. 1976, 1977; Lansman and Haynes 1975; Holz and Stratford 1979). When Ca^{2+} was added to the liposome-protoplast incubation medium or to the protoplasts at the end of a 1.5-h incubation with liposomes still present, protoplast incorporation of liposomes was not increased. However, the pH of the protoplast-liposome incubation medium affected the amount of incorporation of liposomes into protoplasts (Fig. 2). Low pH enhanced liposome-protoplast interaction. When PEG was used to augment fusion with liposomes, greater interaction occurred at the lower pH values. Protoplasts incubated at pH 4.7 and 5.2 showed no loss in viability or capacity to form cell walls and divide when compared to untreated protoplast populations.

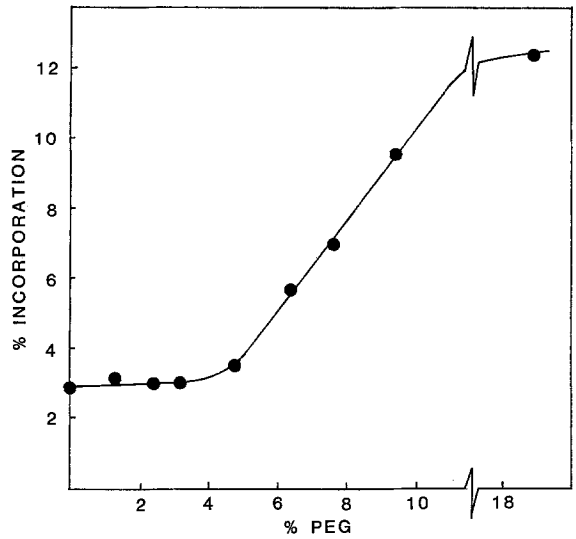

Fig. 1. PEG fusion of liposomes with carrot protoplasts. Protoplasts 120,000/ml and liposomes (A_{650}=0.931) were incubated for 1.5 h at 32° C. Each point represents the average of three experiments

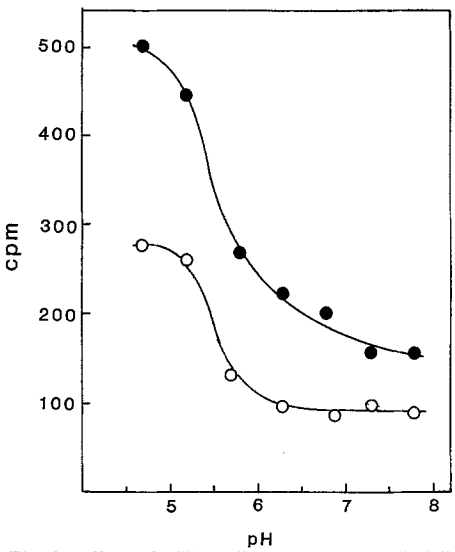

Fig. 2. Effect of pH on liposome-mediated delivery of [^{3}H]DNA to protoplasts in the presence (●) and absence (○) of 18% PEG. Protoplasts (330,000/ml) and liposomes (A_{650}=1.035) were incued for 1.5 h at 32° C. Each point represents the average of at least three experiments

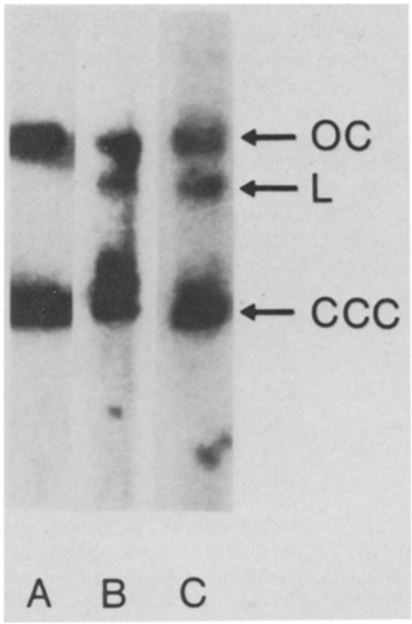

Fig. 3. Plasmid pBR322 DNA sequestered in carrot nuclei. Carrot nuclear DNA was analyzed for the presence of pBR322 DNA by agarose gel electrophoresis, transfer to nitrocellulose, and hybridization as described in Material and Methods. Lane (*A*) depicts a control sample of covalently closed circular (*CCC*) and open circular (*OC*) plasmid molecules. Carrot DNA isolated from nuclei that were (*B*) or were not (*C*) treated with DNase prior to lysis also exhibits linear (*L*) plasmid molecules

Analysis of nuclear-sequestered DNA. Carrot protoplasts were added to liposomes in which pBR322 DNA was encapsulated and were incubated in fusion medium (pH 5.2) for 2 h at 37° C. Nuclei were isolated in the presence of 200 μg/ml rat DNA. The carrier rat DNA was included to minimize aggregation of cytoplasmic-sequestered pBR322 molecules to nuclei upon protoplast lysis. The nuclei were then treated with DNase prior to lysis to remove any non-sequestered DNA copurified with the nuclei. Control experiments (data not shown), in which nuclei were isolated from protoplasts lysed in the presence of exogenous pBR322 DNA, demonstrated that such DNase treatment was necessary.

DNA isolated from carrot nuclei was analyzed by molecular hybridization for the presence and size of pBR322 DNA sequences. An autoradiogram of carrot DNA isolated from nuclei which were (lane B) or were not (lane C) treated with DNase prior to lysis is presented in Fig. 3. It is important to note that the majority of pBR322 molecules detected were autonomous, intact, covalently closed circular (CCC) or open-circular (OC) molecules, although linear (L) molecules reflecting double-strand breaks were also detected. The similarity in intensity of hybridization and degree of plasmid integrity in lanes B and C demonstrate that the pBR322 DNA was sufficiently sequestered within the nuclei to be DNase-resistant under these experimental conditions. The ratio of CCC to OC molecules was observed to be slightly lower in the liposome-delivered pBR322 as compared to the untreated pBR322 preparation used in the experiments (lane A), indicating some nicking of the DNA delivered to the nuclei. We saw no evidence of pBR322 sequences associated with the high-molecular-weight carrot DNA fraction.

Quantitation of the sequestered pBR322 DNA was performed by a comparison to hybridization intensities of varying amounts of pBR322 DNA subjected to electrophoresis in the same gel as in lane A (Fig. 3). This comparison permitted estimation of the weight of sequestered pBR322 DNA in a given gel slot. Since the number of nuclei lysed and applied to the gel slot was determined beforehand by counting in a haemocytometer, the number of plasmid molecules per nucleus could be estimated based on a plasmid molecular weight of $2.7 \cdot 10^6$ dalton. A range of 200–1,000 copies of pBR322 per nucleus were detected in several independent experiments.

Discussion

Liposomes can be used as vehicles for the insertion of macromolecules such as RNA and DNA into plant protoplasts (Matthews et al. 1979; Lurquin 1979, 1981). Our experiments, using molecular hybridization to detect plasmid pBR322 DNA delivered to carrot nuclei under optimum conditions, demonstrate that an average of several hundred copies of a low-molecular-weight DNA plasmid can be delivered to plant nuclei in a relatively undegraded form. Since the nuclei were isolated and analyzed shortly after fusion, the long-term stability of such DNA or its fate in regard to integration or maintenance are unknown.

The use of polyethylene glycol (PEG) to fuse plant protoplasts is well documented in literature (Kao and Michayluk 1974; Constabel and Kao 1974). Therefore, it is not surprising that PEG augments liposome incorporation by carrot protoplasts and also of cowpea protoplasts (Lurquin 1979). Although the interaction of PEG with liposomes and protoplasts is not well understood, it appears that liposomes loosely associated with protoplasts prior to PEG addition become much more tightly associated and fuse upon treatment with PEG. If liposomes do not have a chance to associate with the protoplast membrane during a preincubation period, then PEG addition does not detectably augment liposome-protoplast association.

Our observations and those of Lurquin (1979) are in contrast to those of Uchimiya (1981), which do not indicate an increase in delivery of liposome contents using PEG. However, in that case a fluorescent

dye was used to monitor liposome-mediated delivery. Ostro et al. (1980) have demonstrated rapid loss (50–90% in 2 h) of low-molecular-weight molecules from liposomes. Thus, no increase in delivery would be expected if the fluorescent dye was rapidly lost from the liposomes. Also, Uchimiya used large, multilamellar liposomes which may possess different properties from the unilamellar vesicles utilized in our laboratory. Further experimentation is needed to understand this variation in the results of experiments utilizing polyethylene glycol.

The results of the present experiments and of previous ones investigating liposome-carrot protoplast interaction (Matthews et al. 1979; Ostro et al. 1980) provide a basis for encapsulating and delivering RNA, DNA and other materials to plant protoplasts in amounts deemed necessary for genetic engineering and molecular-biological studies. Experiments to test the stability and biological expression of DNA and intact chromosomes delivered by liposomes are currently in progress.

References

Cohen, C.M., Weissmann, G., Hoffstein, S., Awasthi, Y.C., Srivastava, S.K. (1976) Introduction of purified hexosaminidase A into Tay-Sacks leukocytes by means of immunoglobulin-coated liposomes. Biochemistry **15**, 452–460

Constabel, F., Kao, K.N. (1974) Agglutination and fusion of plant protoplasts by polyethylene glycol. Can. J. Bot. **52**, 1603–1606

Denhardt, D. (1966) A membrane-filter technique for the detection of complimentary DNA. Biochem. Biophys. Res. Commun. **23**, 641–646

Dimitriadis, G.J. (1978) Translation of rabbit globin mRNA introduced by liposomes into mouse lymphocytes. Nature **274**, 923–924

Dimitriadis, G.J. (1979) Entrapment of plasmid DNA in liposomes. Nucleic Acids Res. **6**, 2697–2705

Dudits, D., Hadlaczky, G.Y., Levi, E., Fejer, O., Haydu, Z.S., Lazar, G. (1977) Somatic hybridization of *Daucus carota* and *D. capillifolius* by protoplast fusion. Theor. Appl. Genet. **51**, 127–132

Hoffman, R.M., Margolis, L.B., Bergelson, L.D. (1978) Binding and entrapment of high molecular weight DNA by lecithin liposomes. FEBS Lett. **93**, 365–368

Holz, R.W., Stratford, C.A. (1979) Effects of divalent ions on vesicle-vesicle fusion studied by a new luminescence assay for fusion. J. Membr. Biol. **46**, 331–358

Kao, K.N., Michayluk, M.R. (1974) A method for the high-frequency intergeneric fusion of plant protoplasts. Planta **115**, 355–367

Lansman, J., Haynes, D.H. (1975) Kinetics of a Ca^{+2}-triggered membrane aggregation reaction of phospholipid membranes. Biochim. Biophys. Acta **394**, 335–347

Lurquin, P.F. (1979) Entrapment of plasmid DNA by liposomes and their interactions with plant protoplasts. Nucleic Acids Res. **6**, 3773–3784

Lurquin, P.F. (1981) Binding of plasmid loaded liposomes to plant protoplasts: Validity of biochemical methods to evaluate the transfer of exogenous DNA. Plant Sci. Lett. **21**, 31–40

Maio, J.J., Schildkraut, C.L. (1967) Isolated mammalian metaphase chromosomes I. General characteristics of nucleic acids and proteins. J. Mol. Biol. **24**, 29–39

Mannino, R.J., Allebach, E.S., Strohl, W.A. (1979) Encapsulation of high molecular weight DNA in large unilamellar phospholipid vesicles. FEBS Lett. **101**, 229–232

Matthews, B., Dray, S., Widholm, J., Ostro, M. (1979) Liposome-mediated transfer of bacterial RNA into carrot protoplasts. Planta **145**, 37–44

Matthews, B.F., Widholm, J.M. (1978) Regulation of lysine and threonine synthesis in carrot cell suspension cultures and whole carrot roots. Planta **141**, 315–321

McDonnell, M.W., Simon, M.N., Studier, F.W. (1977) Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**, 119–146

Mukherjee, A.B., Orloff, S., Butler, J. Deb, Triche, T., Lalley, P., Schulman, J.D. (1978) Entrapment of metaphase chromosomes into phospholipid vesicles (lipochromosomes). Carrier potential in gene transfer. Proc. Natl. Acad. Sci. USA **75**, 1361–1365

Ostro, M.J., Giacomoni, D., Lavelle, D., Paxton, W., Dray, S. (1978) Evidence for translation of rabbit globin mRNA after liposome-mediated insertion into a human cell line. Nature **274**, 921–923

Ostro, M.J., Lavelle, D., Paxton, W., Matthews, B., Giacomoni, D. (1980) Parameters affecting the liposome-mediated insertion of RNA into eukaryotic cells in vitro. Arch. Biochem. Biophys. **201**, 392–402

Papahadjopoulos, D., Poste, G., Mayhew, E. (1974) Cellular uptake of cyclic AMP captured within phospholipid vesicles and effect on cell-growth behavior. Biochim. Biophys. Acta **363**, 404–418

Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G., Lazo, R. (1977) Studies on membrane fusion. III. The role of calcium-induced phase changes. Biochim. Biophys. Acta **465**, 579–598

Papahadjopoulos, D., Vail, W.J., Pangborn, W.A., Poste, G. (1976) Studies on membrane fusion. II. Induction of fusion in pure phospholipid membranes by calcium ions and other divalent metals. Biochim. Biopyhs. Acta **448**, 265–283

Poste, G., Allison, A.C. (1973) Membrane fusion. Biochim. Biophys. Acta **300**, 421–465

Rigby, P.W.J., Dieckmann, M., Rhodes, C., Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**, 237–251

Rollo, F., Galli, M., Paresi, B. (1981) Liposome-mediated transfer of DNA to carrot protoplasts: A biochemical and autoradiographic analysis. Plant Sci. Lett. **20**, 347–354

Slavik, N.S., Widholm, J.M. (1978) Inhibition of deoxyribonuclease activity in the medium surrounding plant protoplasts. Plant Physiol. **62**, 272–275

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**, 503–517

Szoka, F., Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc. Natl. Acad. Sci. USA **75**, 4194–4198

Uchimiya, H. (1981) Parameters influencing the liposome-mediated insertion of fluorescein diacetate into plant protoplasts. Plant Physiol. **67**, 629–632

Wahl, G.M., Stern, M., Stark, G.R. (1979) Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**, 3683–3687

Weissmann, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gotlieb, A., Nagle, D. (1975) A general method for the introduction of enzymes, by means of immunoglobulin-coated liposomes, into lysosomes of deficient cells. Proc. Natl. Acad. Sci. USA **72**, 88–92

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